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ACIDIFICATION AND SODIUM ENTRY IN FROG SKIN EPITHELIUM

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Acidification of the external medium by isolated frog skin epithelium (*Rana catesbeiana, Rana temporaria,* and *Caudiververa caudiververa*) and its relationship to Na^+ uptake was studied. Acidification was measured by the pH-stat technique under short-circuit or open-circuit conditions. The results of this study demonstrate that (a) acidification by these species of in vitro frog skins is not directly coupled to Na^+ or anion transport; (b) acidification can be inhibited by the diuretic drug amiloride, but only at high external Na^+ concentrations; (c) acidification rate in these species of frog skin is controlled in part by the metabolic production of CO_2; and (d) the positive correlation between net Na^+ absorption and net acidification observed in whole animal studies could not be replicated in the in vitro skin preparation, even when the frogs were first chronically stressed by salt depletion, a physiological state comparable to that used in the in vivo experiments.

Introduction

A major physiological function of many salt absorbing epithelia is the regulation of extracellular acid-base balance. In a number of cellular and epithelial transport systems, evidence exists that acidification, specifically hydrogen ion secretion, occurs in an obligatory one-for-one exchange with sodium (for reviews, see Refs. 1–3). Furthermore, this Na^+–H^+ exchange process appears susceptible to inhibition by the diuretic compound, amiloride [4–8].

Differences in the molecular mechanism of acidification exists between the two general classes of transporting epithelia, the electrically leaky and tight tissues. In leaky epithelia, like gallbladder and renal proximal tubule, strong evidence exists supporting the presence of an electroneutral Na^+-H^+ countertransport system [7,9–11]. Inhibition of this exchange system requires much higher concentrations (at least two orders of magnitude) of amiloride than the Na^+ entry sites in tight epithelia. In contrast, acidification of the external medium by tissues like the toad or turtle urinary bladder is not dependent upon the presence of Na^+ in the bathing medium [12–18]. However, there does appear to be some electrical coupling between Na^+ and H^+ fluxes in the toad bladder [15]. Ludens and Fanestil [17] reported that acidification by the toad bladder was not inhibited by amiloride. Husted and Steinmetz [19], however, found that amiloride could inhibit acidification by the turtle bladder, and further proposed that this inhibition resulted from a hyperpolarization of the luminal cell membrane.

In the short-circuited frog skin preparation, no dependence of the rate of acidification on external Na^+ and no inhibition of acidification by amiloride has ever been reported [6,20–22]. However, in whole frogs or in in vitro *Rana esculenta* skins in the open circuit and bathed in solutions of low Na^+ concentration, net Na^+ absorption by the skin was found to be stoichiometrically equivalent to net H^+ efflux [6,23–

Abbreviations: ADH, antidiuretic hormone; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

25]. Furthermore, both Na^+ absorption and H^+ secretion were inhibited by amiloride. Emilio and Menano [21] concluded that there are species differences among short-circuited amphibian skins with regard to the mechanism of acidification, inasmuch as 10^{-4} M amiloride could inhibit 50–60% of the total acidification in the toad skin, and was without effect in skins from *Rana ridibunda.*

The present study was undertaken using in vitro frog skin epithelium as a model system with three questions in mind. First, what is the molecular mechanism of acidification, and is this process indeed independent of external Na^+? Second, what is the nature of the amiloride inhibition of acidification? Third, what are the reasons for the discrepancy between the in vivo and in vitro frog skin with regard to acidification? The results show that acidification of the external medium by the isolated frog skin is independent of external Na^+ either in Cl^- or SO_4^{2-} medium, under open- or short-circuit conditions. In contrast to literature reports cited above, amiloride could inhibit up to 60% of the acidification rate, but only when the external sodium was high. The apparent discrepancy in the mechanism of acidification between whole frogs and the isolated skin cannot be accounted for by the pre-experimental history of the animals. Acidification by the skins isolated from salt-depleted frogs is not Na^+-dependent, and, again, is inhibited by amiloride only at high concentrations of external Na^+.

Methods

The abdominal skin of the bullfrog, *Rana catesbeiana* (West Jersey Biological Supply Co. Wenonah, NJ), the Northern European grassfrog, *Rana temporaria* (Charles D. Sullivan Company, Nashville, TN), or the Chilean bullfrog, *Caudiververa caudiververa* (formerly *Calytocephalella gayi*) was mounted as a 3.14 cm^2 flat sheet. The skin was placed between Lucite half-chambers, equipped with glass bubble-lift solution reservoirs. The solution in each chamber (12 ml each) were circulated and simultaneously aerated with 100% O_2 from which any trace CO_2 was removed by passage through two 5 M NaOH traps. In some experiments, the serosal solution was bubbled with either 99% O_2/1% CO_2 or 95% O_2/5% CO_2. All experiments were performed at room temperature (19°C). The Chilean frogs were a gift from Dr. Osvaldo Alvarez, Departamento de Biologia, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

The open-circuit potential across the skin was measured with calomel electrodes, and current was passed through the skin via Ag-AgCl electrodes. The outside bathing solution always served as reference. An automatic voltage clamp that compensated for the resistance of the solution between the voltage-sensing electrodes was used to pass the appropriate current through the skin to clamp the transepithelial potential to 0 mV. The magnitude of the short-circuit current (I_{sc}) and net active sodium transport was equivalent for *R. catesbeiana* and *R. temporaria* under all conditions utilized in these experiments [26,27]. The same was assumed to be true for *C. caudiververa,* Since 10^{-4} M amiloride completely inhibited I_{sc} under all experimental conditions.

The composition of the standard chloride Ringer solution was 110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 5 mM Hepes buffer, pH 7.3. The composition of the standard sulfate Ringer solution was 55 mM Na_2SO_4, 55 mM mannitol, 1.25 mM K_2SO_4, 1.0 mM $CaSO_4$, 5 mM Hepes (pH 7.3). The internal (or serosal) solution contained one of these solutions at all times. Several different Ringer solutions were used in the external chamber, depending upon the nature of the experiment to be performed. All external solutions were unbuffered except for a small quantity (0.3 mM) of Na_2HPO_4 or K_2HPO_4. Furthermore, all external solutions contained 1.0 mM Ca and 2.5 mM K (either as the chloride or sulfate). When high Na solutions were used, the Ringer contained either 110 mM NaCl or 55 mM Na_2SO_4 plus 55 mM mannitol. In experiments in which the external sodium concentration was varied, the initial solution contained only 0.3 mM HPO_4^{2-}, 1.0 mM Ca^{2+}, and 2.4 mM K^+. The sodium concentration was increased stepwise by addition of microliter volumes of concentrated NaCl or Na_2SO_4 solutions. All chemicals were reagent grade, and were obtained from either Sigma or J.T. Baker Co. Amiloride was a gift from Merck, Sharp & Dohme Research Laboratories, West Point, PA. When ouabain or DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, Calbiochem) were added to the solutions bathing the frog skin, they were added 45–60 min prior to determination of acidification rate. When DIDS was used, the experiments were performed in

the dark and the compound was present throughout the experiments. Solutions of both DIDS and ouabain were made up fresh for each experiment.

In order to salt-deplete frogs, bullfrogs were kept for at least 14 days in a large glass aquarium at room temperature in distilled, deionized H_2O. The frogs were washed and the water replaced twice daily.

Acidification of the external solution by the frog skin was measured by the pH-stat technique [12]. The pH-stat assembly consisted of a Radiometer pH meter (Model 26), TTT60 titrator, and an autoburette (ABU11) containing 0.075 mM NaOH as a titrant. A combination glass electrode (Radiometer model GK 2321C) and polyethylene delivery tube were mounted directly in the external solution. The maximum concentration change of sodium in the bathing medium produced during any given titration period was no more than 10 μM. All solution Na^+ concentrations were routinely measured by atomic absorption spectroscopy (Perkin-Elmer, Model 5000). The net rate of acidification produced by the frog skin was computed from the slope of the graph of volume of titrant added versus time. Measurement of acidification rate was conducted for at least 20 min under each experimental condition. The slope was computed by linear regression analysis. Over 95% of the total number of experiments had slopes with a correlation coefficient greater than 0.95; the remaining 5% had slopes below 0.90 and were not used in the computations.

Probabilities that the difference between two sample means were significant were assessed by computing the *t*-statistics for the difference between paired or unpaired means. When the significance between three or more population means needed to be determined, one-way analysis of variance was employed. All results are expressed as mean ±1 S.E. of the mean.

Results

Properties of the acidification mechanism in in vitro frog skin

If acidification of the external medium proceeds by a coupled Na^+-H^+ exchange mechanism, a diminution in acidification rate should occur when Na^+ is removed from the external medium. To test this hypothesis three different experimental procedures were followed. First, in order to see if there is any relationship between acidification and net Na^+ influx, the spontaneous I_{sc} measured in 67 *R. catesbeiana* skins was plotted against the simultaneously measured acidification rate (Fig. 1). There was no correlation between these two parameters. The same observation held for *R. temporaria* and *C. caudiververa* skins (data not shown).

A more direct evaluation of the dependence of acidification on external $[Na^+]$ is shown in Fig. 2. As external Na^+ was raised, I_{sc} increased following a saturating curve of the type previously described [28]. The rate of acidification, however, was essentially independent of external sodium. The rate of acidification (expressed as equivalents of H^+) at 110 mM Na^+ was slightly lower than that observed at 0 mM Na^+ (7.2 ± 1.0 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1} versus 11.3 ± 1.2 nmol $\cdot$ cm^{-2} $\cdot$ h^{-1}, respectively, $P < 0.05$). This lack of positive dependence of acidification rate on external Na^+ was seen in all species of frog skin bathed in either Cl^- or SO_4^{2-} media, under open- or short-circuit conditions.

Since the frog skin initially separated asymmetric solutions, Na^+ could have diffused from the serosal to the apical bathing solution, possibly saturating a high affinity, Na^+-H^+ exchange system at very low (<1 mM) external $[Na^+]$. To check this, the experiments presented in Fig. 2 were repeated using symmetrical sodium solutions (recrystallized choline chloride as

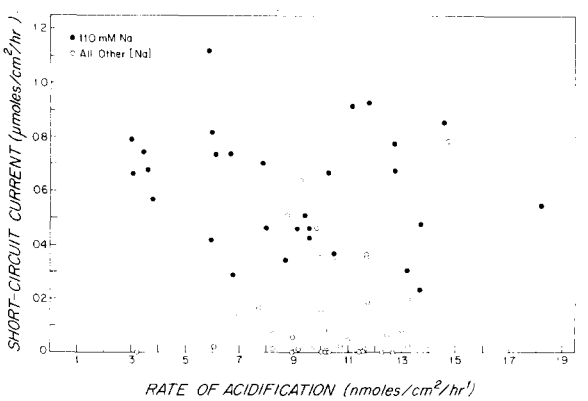

Fig. 1. Spontaneous short-circuit current plotted against the rate of acidification simultaneously measured in 67 individual *R. catesbeiana* skins. The solid circles were obtained from skins bathed with 110 mM Na^+ (either Cl^- or SO_4^{2-}) on both sides, while the open circles were from skins initially bathed with either 0, 1, 3, 5, 20, or 55 mM Na^+ externally.

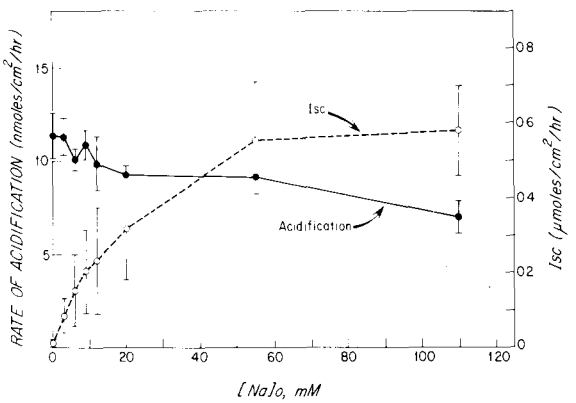

Fig. 2. The dependence of I_{sc} and acidification rate on external Na^+ concentration in isolated *R. catesbeiana* skin. Each point represents the mean of three experiments, while the verticle line is one standard error of the mean.

replacement). Na^+ concentrations in nominally Na^+-free media were determined with atomic absorption spectroscopy to be in the range 1–2 μM. The results, however, were the same. The rate of acidification observed at 0 mM Na^+ was 16.1 ± 0.8 nmol · cm^{-2} · h^{-1} while that measured at 55 mM was 12.9 ± 1.6 nmol · cm^{-2} · h^{-1}, values not significantly different from one another ($P > 0.1$). In addition, 0.1 mM increments in external [Na^+] from 0 to 1.5 mM with concomitant acidification measurements revealed no dependence of acidification on external sodium in this range of concentrations.

In all of these experiments, the external K^+ and Ca^{2+} concentrations were held constant. It is therefore possible that acidification may occur as a result of a K^+ or Ca^{2+}-H^+ exchange system. Hence, it was important to examine the external cation specificity of the acidification process. The results of these experiments are summarized in Table I. There was no significant difference ($P > 0.5$) in the rate of acidification under conditions when the major external cation was Na^+, choline$^+$, or when the ionic strength was allowed to vary (no replacement). Using either K^+, Li^+, Rb^+, or Cs^+ to replace Na^+, a significant, albeit slight, depression of acidification rate was observed. The rates of acidification observed under these conditions were not significantly difference from each other ($P > 0.5$). Similar results were obtained in Ca^{2+}-free solutions.

A series of experiments was designed to compare

TABLE I

THE EFFECT OF EXTERNAL CATION REPLACEMENT ON ACIDIFICATION RATE OF ISOLATED BULLFROG SKIN EPITHELIA ($N = 4$)

Ion (110 mM)	Rate of acidification (nmol · cm^{-2} · h^{-1})
Na^+	14.7 ± 1.24
Choline$^+$	10.9 ± 1.98
K^+	8.82 ± 0.96
Li^+	8.64 ± 0.46
Rb^+	9.21 ± 1.28
Cs^+	9.82 ± 0.51
No replacement	12.0 ± 0.74

the rate of acidification as a function of transepithelial potential difference in 110 mM NaCl Ringer solution. In paired experiments, no significant difference between the rate of acidification measured in open circuit (17.2 ± 2.04 nmol · cm^{-2} · h^{-1}) or under short-circuit (15.8 ± 1.94 nmol · cm^{-2} · h^{-1}) conditions was noted ($P > 0.5$; $N = 6$). Further, in three additional experiments, clamping the transepithelial potential difference to either −50 mV or +100 mV left acidification rate unchanged with respect to open- or short-circuit conditions (rate of acidification in nmol · cm^{-2} · h^{-1}: open = 11.8 ± 3.8; short-circuit = 9.7 ± 3.3; −50 mV = 11.5 ± 2.7; +100 mV = 9.9 ± 3.6, $P > 0.5$).

Fig. 3 displays the external pH-dependence of

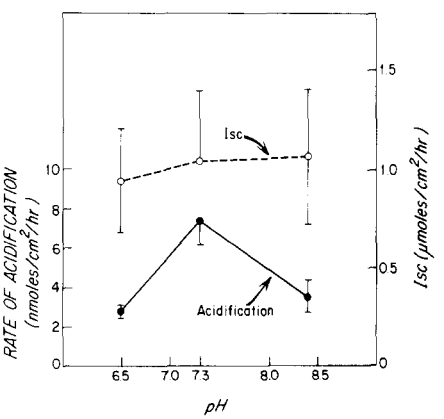

Fig. 3. The effect of pH on I_{sc} and acidification rate in *R. catesbeiana* skin. Each point is the mean of three experiments, ±1 S.E.M.

both I_{sc} and acidification rate in isolated bullfrog skin. I_{sc} was independent of external pH (6.4–8.5), as has been previously observed [28]. The rate of acidification, however, was highly influenced by pH, falling abruptly when the pH was either increased or decreased from 7.3 ($P < 0.005$, from pH 6.5 to 7.3 and $P < 0.05$ from pH 8.5 to 7.3).

The steady-state rates of acidification by in vitro bullfrog skin are at least one order of magnitude lower than those reported for other acidifying epithelia, for example, turtle urinary bladder [19] or rabbit proximal tubule [29]. It has also been shown that the frog skin is capable of actively transporting chloride at low external Na^+ concentration [30,31]. It is therefore possible that external acidification may be attenuated by HCO_3^- secretion (via a Cl^--HCO_3^- exchange). To examine this possibility the effects of an inhibitor of anion transport (DIDS) was studied on acidification rate and I_{sc} at low and high Na^+. The results of these experiments are summarized in Table II. When $[Na]_o = 110$ mM, 10^{-4} DIDS had no significant effect on either I_{sc} ($P > 0.2$) or acidification rate ($P > 0.5$). Amiloride (10^{-4} M) inhibited I_{sc} by 98% and acidification by 47% after DIDS treatment. There was no significant difference between the amiloride-induced inhibition of I_{sc} and acidification rate after DIDS treatment as compared with control, i.e., no DIDS treatment (Fig. 4). Likewise, DIDS did not affect I_{sc} or acidification when $[Na]_o = 6$ mM (Table II).

TABLE II

THE EFFECT OF 10^{-4} M DIDS ON I_{sc} AND ACIDIFICATION RATE OF ISOLATED BULLFROG SKIN

Condition	I_{sc} (μmol · cm^{-2} · h^{-1})	Acidification rate (nmol · cm^{-2} · h^{-1})
110 mM Na^+ ($N = 4$)	0.767 ± 0.340	8.47 ± 2.02
+DIDS	0.814 ± 0.280	10.7 ± 1.91
+Amiloride (10^{-4})	0.012 ± 0.007	5.62 ± 2.29
6 mM Na^+ ($N = 5$)	0.211 ± 0.146	9.45 ± 2.52
+DIDS	0.189 ± 0.074	8.45 ± 2.62
+Amiloride (10^{-4})	−0.021 ± 0.048	8.19 ± 2.71

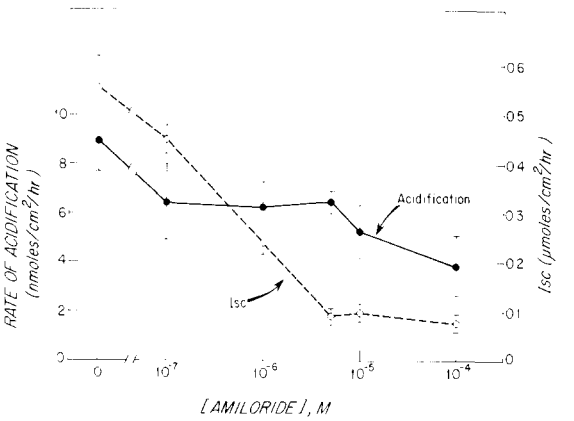

Fig. 4. Log-dose curves of the effect of amiloride on I_{sc} and acidification in *R. catesbeiana* skin at 110 mM external Na^+. Each point is the average value of six experiments.

The effect of amiloride and other compounds on the acidification rate

The effect of the diuretic drug amiloride on I_{sc} and acidification rate was examined at high (Fig. 4) and low (Fig. 5) external $[Na^+]$. Fig. 4 is a plot of both absolute rate of acidification and I_{sc} as a function of the external amiloride concentration. Amiloride inhibited both I_{sc} and acidification. Acidification was inhibited by an average of 25% with 10^{-7} to 10^{-5} M amiloride. Higher amiloride concentrations, while inhibiting I_{sc} over 90%, inhibited acidification rate by a maximum 60%. Fig. 5 summarizes a similar set of experiments, only at an external $[Na^+]$ of 6 mM. It is apparent that acidification rate was unaffected at all amiloride concentrations when $[Na^+]$ is low.

The effects of a number of other compounds and experimental manipulations on acidification rate are summarized in Table III. The results are expressed as the steady-state acidification rate observed after experimental treatment normalized to the control value of acidification rate measured just prior to treatment. It can be seen from this table that addition of antidiuretic hormone or insulin had no effect upon the rate of acidification by the isolated frog skin. On the other hand, 10^{-4} M ouabain significantly ($P <$ 0.005) inhibited acidification rate, as did bubbling the bathing solution with 100% N_2 ($P < 0.001$). Ouabain treatment inhibited I_{sc} by over 90% in all experiments, while N_2 bubbling resulted in an average

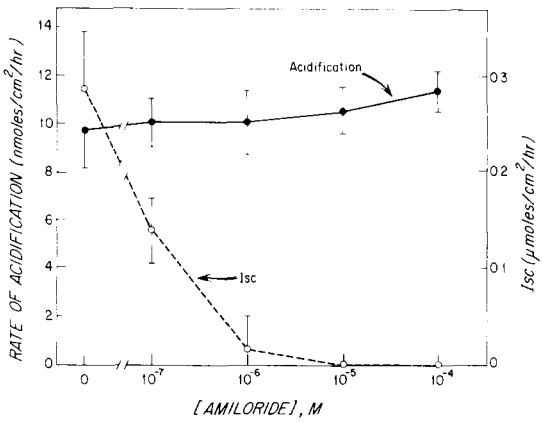

Fig. 5. Log-dose response curves of the effect of amiloride on I_{sc} and acidification rate in *R. catesbeiana* skin bathed with 6 mM Na outside. Each point is the average from four experiments.

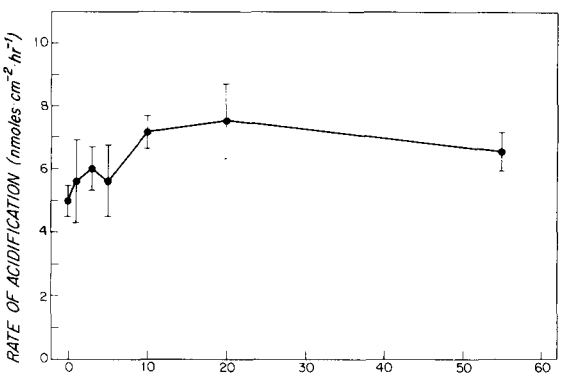

Fig. 6. A summary of experiments in which the rate of acidification of isolated *C. caudiververa* skin was measured as a function of external Na^+ concentration. The skins were taken from seven chronically salt depleted frogs, and performed in the open circuit.

62% inhibition of I_{sc}. A 30-min serosal exposure to the carbonic anhydrase inhibitor, acetazolamide (5 mM), resulted in an average 34% inhibition of acidification. Bubbling the serosal solution with 1% CO_2 stimulated acidification by 89%. 5 mM deoxyglucose inhibited acidification rate by 48%. The addition of 0.05 mM dinitrophenol, an uncoupler of oxidative phosphorylation, stimulated acidification by 24% ($P < 0.02$). Furthermore, apical addition of 10^{-4} M amiloride to dinitrophenol-treated frog skin at 110 mM Na^+ (data not shown) resulted in only 11.3 ± 4.8% ($N = 4$) inhibition of acidification rate, a value barely reaching significance ($P < 0.1$).

TABLE III

THE EFFECT OF VARIOUS COMPOUNDS AND DIFFERENT TREATMENTS ON ACIDIFICATION RATE IN ISOLATED BULLFROG SKIN EPITHELIUM

Experimental condition	*N*	Rate of acidification (experimental/control ± S.E.)
0.1 mM ouabain (serosal)	4	0.72 ± 0.04
2 mU/ml ADH (serosal)	4	1.07 ± 0.09
2 mU/ml insulin (serosal)	3	0.99 ± 0.02
5 mM acetazolamide (serosal)	3	0.66 ± 0.09
5 mM 2-deoxyglucose (serosal)	5	0.52 ± 0.09
0.05 mM DNP (serosal)	4	1.24 ± 0.15
100% N_2 (both sides)	4	0.69 ± 0.11
1% CO_2 (serosal)	5	1.89 ± 0.22

Acidification by isolated frog skin obtained from salt-depleted frogs

Fig. 6, like Fig. 2, plots acidification rate versus the external Na^+ concentration, only in this case from the skin epithelia isolated from Chilean frogs (*C. caudiververa*) previously salt depleted. Acidification again appeared not to be dependent upon external Na^+. This experiment was performed on skins obtained from seven animals, all with similar results. It may be concluded that salt depletion per se does not activate latent or promote synthesis of apical Na^+-coupled, H^+ translocating systems.

Discussion

A sodium-hydrogen exchange reaction has been implicated in many important physiological functions such as fertilization [4], intracellular pH regulation [32], cell volume regulation [8], and insulin action on glycolysis [33]. In all of the aforementioned cases, amiloride has been shown to block this exchange system. In this present paper, a reinvestigation of the apical acidification process by the in vitro frog skin and the effect of amiloride on this mechanism was undertaken. Further, the hypothesis that Na^+-H^+ exchange could be induced by stress (i.e., salt depletion) was tested.

Acidification by the isolated skin epithelium of *R. catesbeiana, R. temporaria* or *C. caudiververa* is not

correlated with steady-state levels of I_{sc} (Fig. 1), and is independent of the external sodium ion concentration (Fig. 2). Further, there does not seem to be any major dependence of acidification rate on external cations (Table I). Although Na^+ substitution with K^+, Li^+, Rb^+, or Cs^+ produced a small diminution in the absolute rate of acidification as compared to that measured in Na^+, choline$^+$, or dilute media (Table I), no particular trend could be noted. In fact, acidification proceeds unabated when the external K^+ concentration is varied from 0 to 25 mM (data not shown). These results, therefore, preclude the existence of any cation-hydrogen exchange systems, particularly Na^+-H^+ or K^+-H^+ exchanges [34].

The rate of acidification also was not sensitive to changes in transepithelial potential. This is in contrast to the situation which obtains in toad or turtle urinary bladder where acidification rate was found to be a linear function of potential [18,35]. The acidification process by the frog skin was depressed by either increasing or decreasing the pH of the external medium (Fig. 3).

What, then is the mechanism by which the isolated frog skin acidifies the external medium? From the results presented in this paper, it may be concluded that there is no ionic exchange process in the apical membrane. The independence of acidification rate on transepithelial potential difference makes it likely that electrogenic transfer of H^+ across the apical membrane, if present, is not voltage sensitive. Also, the possibility of a coupled HCl secretory process cannot be excluded. The isolated frog skin, under conditions free of exogenous bicarbonate and carbon dioxide, seems to be comparable to the urinary bladder of the turtle in that acidification may ultimately be regulated by the rate of metabolic CO_2 production [13,14]. The observed decrease in acidification rate with ouabain and the stimulation of acidification by dinitrophenol (Table V) may be understood as resulting from changes in metabolic CO_2 production. The carbonic anhydrase inhibitor, acetazolamide, inhibited acidification by 34%, suggesting that a catalyzed, intracellular CO_2 hydration reaction is able to account for at least part of the observed rate of acidification.

The observation that amiloride can inhibit a very large percentage of the total acidification rate at high external Na^+ (Fig. 4), but not when the external Na^+ is low (Fig. 5) is intriguing particularly since the absolute rate of acidification is essentially independent of external Na^+. The inhibition of acidification by amiloride at high external Na^+ can be understood as resulting from three possible causes. First, amiloride may be inhibiting acidification indirectly by decreasing metabolic CO_2 production through reduced net transepithelial Na^+ transport. However, metabolism, as assayed by O_2-consumption measurements in frog skin, decreases to the same extent when either external Na^+ is lowered, or when amiloride is added [36]. Hence, the same decrement in H^+ secretion would be expected to occur when [Na^+] is lowered or when amiloride added. This was not observed. This does not exclude the possibility that in frog skin acidification may be largely supported by hexose monophosphate shunt activity [37], and that the activity of this metabolic pathway shifts at different external Na^+ concentrations. Also, amiloride has been shown to influence glucose metabolism through this shunt pathway [38].

A second possibility is that amiloride may be inhibiting H^+-secretion by hyperpolarizing the luminal cell membrane and hence opposing the movement of protons in the active transport pathway, as proposed by Husted and Steinmetz [19]. If this were true, one would expect that the effects on acidification of lowered external Na^+ should be the same as that of amiloride, since both hyperpolarize the apical cell membrane in frog skin [39,40]. Also, a dependence of acidification on transepithelial voltage clamping level would be predicted. However, neither was observed.

Thirdly, amiloride may have a direct inhibiting action on the acidification process independent of its effect on Na^+ transport. For this to be true, the interaction between amiloride and this secretory mechanism would have to be dependent upon external Na^+, perhaps the affinity of the reaction being enhanced at higher Na^+. An elucidation of this hypothesis must await further experimentation.

It is not apparent why amiloride can inhibit acidification in the species of frog skin used in the present experiments, and not in other species like *R. ridibunda* [21]. The fact that the absolute magnitude of the acidification rate by bullfrog skin is some 5–15-times smaller than that of other acidifying epithelia supports the notion that there in fact may be species

differences in the actual molecular mechanism of acidification, and hence amiloride susceptibility. This idea is further supported by the observed differential sensitivities of the acidifying process among these various epithelia to pharmacological agents such as ADH and ouabain as well as to changes in transepithelial membrane potential.

It is puzzling why a correlation between Na uptake and hydrogen secretion cannot be demonstrated in the isolated frog skin preparation, but seems to be present in whole animal studies [24,25]. The only apparent difference between the in vivo and previous in vitro studies has been the pre-experimental history of the frogs. In the whole animal studies cited above, frogs were salt depleted for 1–2 weeks prior to net flux measurements so that changes in the medium Na^+ and H^+ concentrations could be magnified. It was reasoned that the stress of salt depletion may induce a tightly-coupled Na^+-H^+ exchange system. This was tested by first salt depleting frogs for two weeks and then by isolating the skin epithelium from these animals. However, the rate of acidification was again not influenced by external Na^+ (Fig. 6), even under open circuit conditions in the presence of 25 mM HCO_3^- and 5% CO_2 added serosally, conditions where the vitro *R. esculenta* skin has been shown to possess an amiloride-sensitive Na^+-H^+ exchange system [6]. It is also unlikely that the connective tissue layers or gland cells are responsible for a Na^+-independent acidification process since Emilio and Menano [21] have shown that acidification was identical in Cl^- or SO_4^{2-} media, Na^+ or Na^+-free media, and was not stimulated by ADH in split frog skin (i.e., epidermis separated from the corium by collagenase treatment). The discrepancy between the in vitro and in vivo frog skin, with regard to Na^+-H^+ exchange, therefore, remains unresolved.

In conclusion, acidification of the external medium, by isolated frog skin epithelium is not correlated to the net Na^+ influx, is dependent upon metabolism, occurs as a result of H^+ secretion, and is not modulated by secretion of HCO_3^-. The diuretic drug amiloride can inhibit acidification, but only when the external Na^+ is high. This action appears to result from a direct interaction with the H^+ translocating mechanism. Finally, the discrepancy between the coupling of net Na^+ influx to net H^+ efflux in whole frogs as compared to isolated frog skin epithelium, cannot be explained on the basis of adaptation to the chronic stress of salt depletion.

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